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Regulation of Breast Cancer Stem Cell Trafficking and Metastasis  
by Brain Originated Signals

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14. ABSTRACT <p>The hypothesis of this study was that adrenergic signaling contributes to reducing the synthesis of CXCL12 by stromal cells, thereby promoting the release of breast tumor-initiating cells in the circulation and that the sympathetic nervous system controls the retention of breast cancer stem cells to their niche through the regulated expression of CXCL12.</p> <p>To address this hypothesis, we examined the role of CXCR4<sup>+</sup> CXCL12 in breast cancer stem cell mobilization of GFP-MDA-MB-231 cells in the presence or absence of AMD3100. AMD3100 is a specific antagonist for CXCR4.</p> <p>The routine identification of single tumor cells in the bone marrow using H&amp;E stain, proved difficult. GFP positive cells could be detected in the peripheral blood only if these cells are injected i.v. at 10<sup>6</sup> cells at least. Therefore, the trafficking of cancer stem cells from the mammary gland to the circulation is a rare event that can over a long period of time.</p>					
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## **Final Progress Report**

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### **INTRODUCTION:**

The hypothesis of this study was that adrenergic signaling contributes to reducing the synthesis of CXCL12 by stromal cells, thereby promoting the release of breast tumor-initiating cells in the circulation (1).

To address this hypothesis, first we analyzed the expression levels of CD44<sup>+</sup>/CD24<sup>-</sup> cells in the MDA-MB-231 cells, as a model system for tumor breast cancer cells which express high levels of CD44<sup>+</sup>/CD24<sup>-</sup> cells, by immunostaining using specific antibodies for CD44 and CD24 respectively. As shown in Table 1, the majority of MDA-MB-231 cells contain a subpopulation of CD44<sup>+</sup>/CD24<sup>-</sup> cells (80-90%), unlike MCF-10 A cells, an immortalized normal mammary epithelial cells. These findings are in agreement with other reports (2). When MDA-MB-231 cells were grown in the mammary fat pad of nude mice, similar levels of CD44<sup>+</sup>/CD24<sup>-</sup> subpopulation were observed (about 85%  $\pm$  6%) (2), indicating that there is no increase in this population of cells during tumorigenesis process using this model system.

Next, we wanted to examine the GFP-MDA-MB-231 in the peripheral blood (PB) circulation based on sorting CD24<sup>-</sup> and CD44<sup>+</sup> positive cells. The main problem in this set of experiments is the specificity of CD44 detection in tumor cells since CD44 is expressed in many other cell types. Therefore, there is a need to use EpCAM antibody to select first for epithelial/cancer cells and then analyzed for CD44<sup>+</sup> and CD24<sup>-</sup>. However, using EpCAM marker for epithelial cells, the most primitive cancer stem cells may be left out of the analysis as these cancer stem cells are EpCAM negative, and breast cancer stem cells do not express EpCAM marker. Thus, these detected cells in the blood circulation as CD44<sup>+</sup>/CD24<sup>-</sup> cells still may not represent the “real” cancer stem/progenitor cells in the peripheral blood.

Therefore, we employed GFP-MDA-MB-231 cells where breast cancer cells are tagged to GFP for detection in the PB. We examined the mobilization of tumor cells in the circulation by administration of GFP-tagged MDA-MB-231 cells. We have administered GFP-MDA-MB-231 cells to female nude mice into the mammary fat pads, at 10<sup>6</sup> cells or no cells as a control. After 10 days or 24 days, mice received intraperitoneal single injection of either AMD3100 (5mg/kg) or  $\beta$ 2-adrenergic agonist (5mg/kg), or vehicle control. These time points were chosen based on the effects of these compounds on hematopoietic stem cells and other reports. After six hours, the mice were analyzed for GFP positive cells. We could not detect any GFP cells in the blood (data not shown). Next, we examined the percentage of CD44<sup>+</sup> CD24<sup>-</sup> cells in the blood. As shown in Figure 1 and Figure 2 and summarized in Figure 3, at day 10 there was some CD44<sup>+</sup> CD24<sup>-</sup> cells detected in mice with tumor cell injection in the mammary fat pads and the percentage of cells did not change significantly at day 10 following treatment with  $\beta$ 2-adrenergic agonist or AMD3100 (see Figures 2-3). Interestingly, at day 24, the percentage of CD24<sup>-</sup>/CD44<sup>+</sup> cells was significantly reduced as compared to day 10. Further, mammary fat pad at day 10 showed increased tumor cells in the mammary fat pads of mice administered with GFP-MDA-MB-231 cells (Figure 4B), as compared to mice administered with  $\beta$ 2-adrenergic agonist (Figure 4C) or with AMD3100 (Figure 4D), as compared to control (Figure 4A). Thus, these results suggest mobilization of tumor cells from the tumor site to the tumor niche, but not to the blood circulation.

**Table 1: Expression of CD44<sup>+</sup>/CD24<sup>-</sup> in breast cancer cells by Immunostaining**

<b><u>Cells</u></b>	<b><u>% of CD44<sup>+</sup>/CD24<sup>-</sup></u></b>
<b>MDA-MB-231</b>	80 – 90%
<b>MCF-10A</b>	10 – 15%

### **Expression of SDF-1 $\alpha$ by stroma cells**

The stroma cells within the tumors are known to secrete SDF-1 $\alpha$  while the tumor cells express CXCR4 as noted by us and others (Fig. 5) (see Ref. 1). Treatment with AMD3100 inhibitor, which inhibits the binding of CXCR4 to SDF-1 $\alpha$ , or with  $\beta$ -adrenergic agonist did not result in increased circulation of CD44<sup>+</sup>/CD24<sup>-</sup> cells (Figure 3-4), which may suggest that the process of migration of tumor cell into the circulation is a complex

process regulated by many pathways and that this process of tumor cell trafficking might be CXCR4/ SDF-1 $\alpha$  and  $\beta$ -adrenergic agonist independent.

To exclude the possibility that the lack of tumor cell trafficking is not due to a lack of CXCR4 expression in tumor cells and to better study the regulation of CXCR4 expression by SDF-1 $\alpha$  in MDA-MB-231 cells, we examined the expression levels of CXCR4 in the cell surface of tumor cells and the total expression levels of CXCR4. As shown in Fig. 5, decrease in CXCR4 expression on the cell surface was noted while the total expression by Western blot of CXCR4 was unchanged (Fig. 5B).

Further, SDF-1 $\alpha$  increased the migration of MDA-MB-231 cells (Fig. 6) indicating the functionality of CXCR4 in these cells, which was inhibited by AMD3100 in a dose dependent manner (Fig. 6). SDF-1 $\alpha$  activation of MDA-MB-231 resulted also in increased phosphorylation of pErk1,2 (Fig. 7B) and phospho-AKT (Fig. 7A) indicating further the activation of this pathway in tumor cells.

Thus, although the CXCR4/SDF-1 $\alpha$  is active in tumor cells, we have no evidence on the role of this pathway in circulation of tumor cells in the blood.

#### **KEY RESEARCH ACCOMPLISHMENTS:**

- Additional determinants may play a role in trafficking of cancer stem cells to the peripheral blood (see Ref. 4).
- The trafficking of cancer stem cells from the mammary gland to the circulation needs to be based on specific reagents that detect only the tumor cells and not other CD44<sup>+</sup> cells in the circulation.

**Figure 1**

Day 10

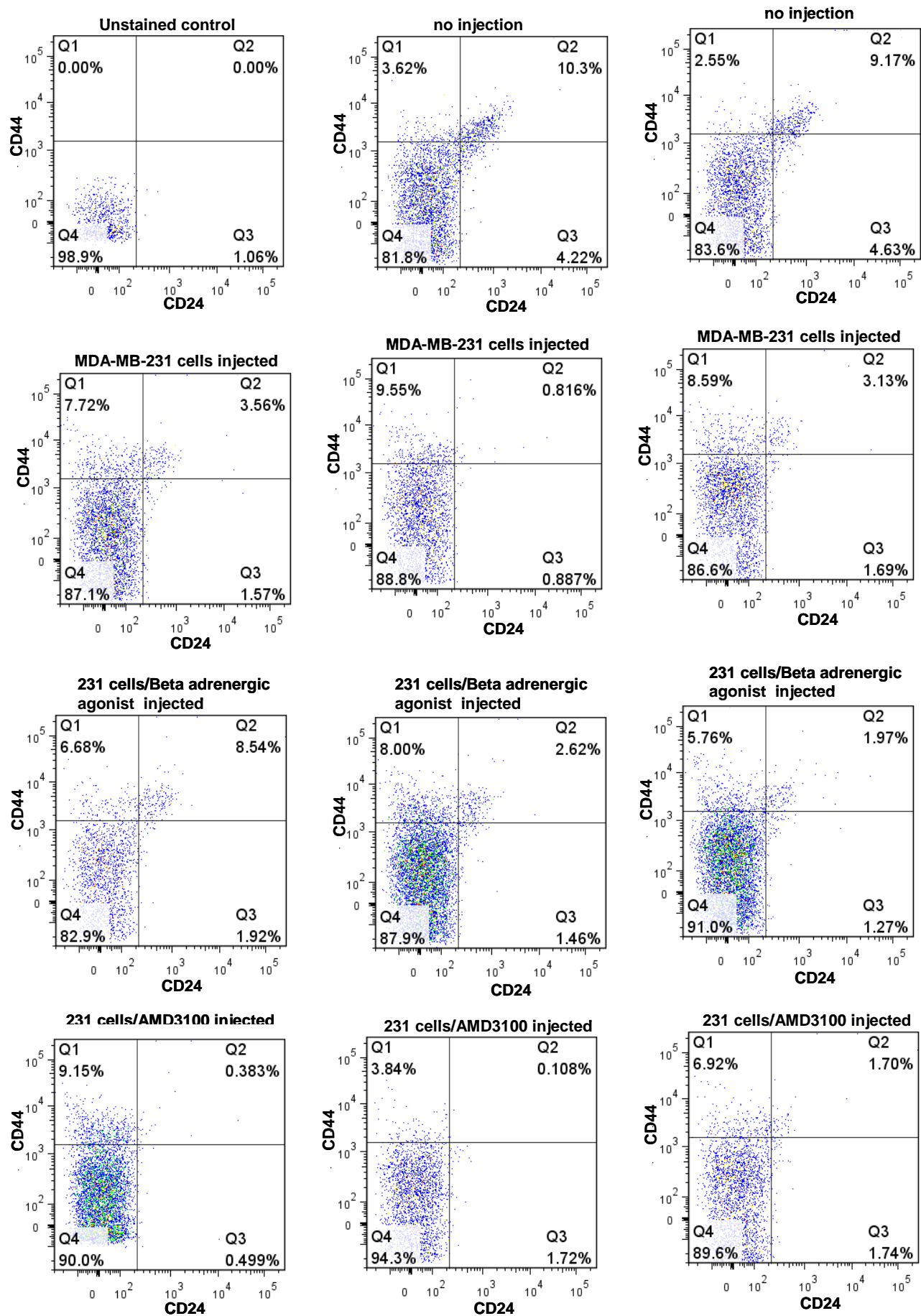
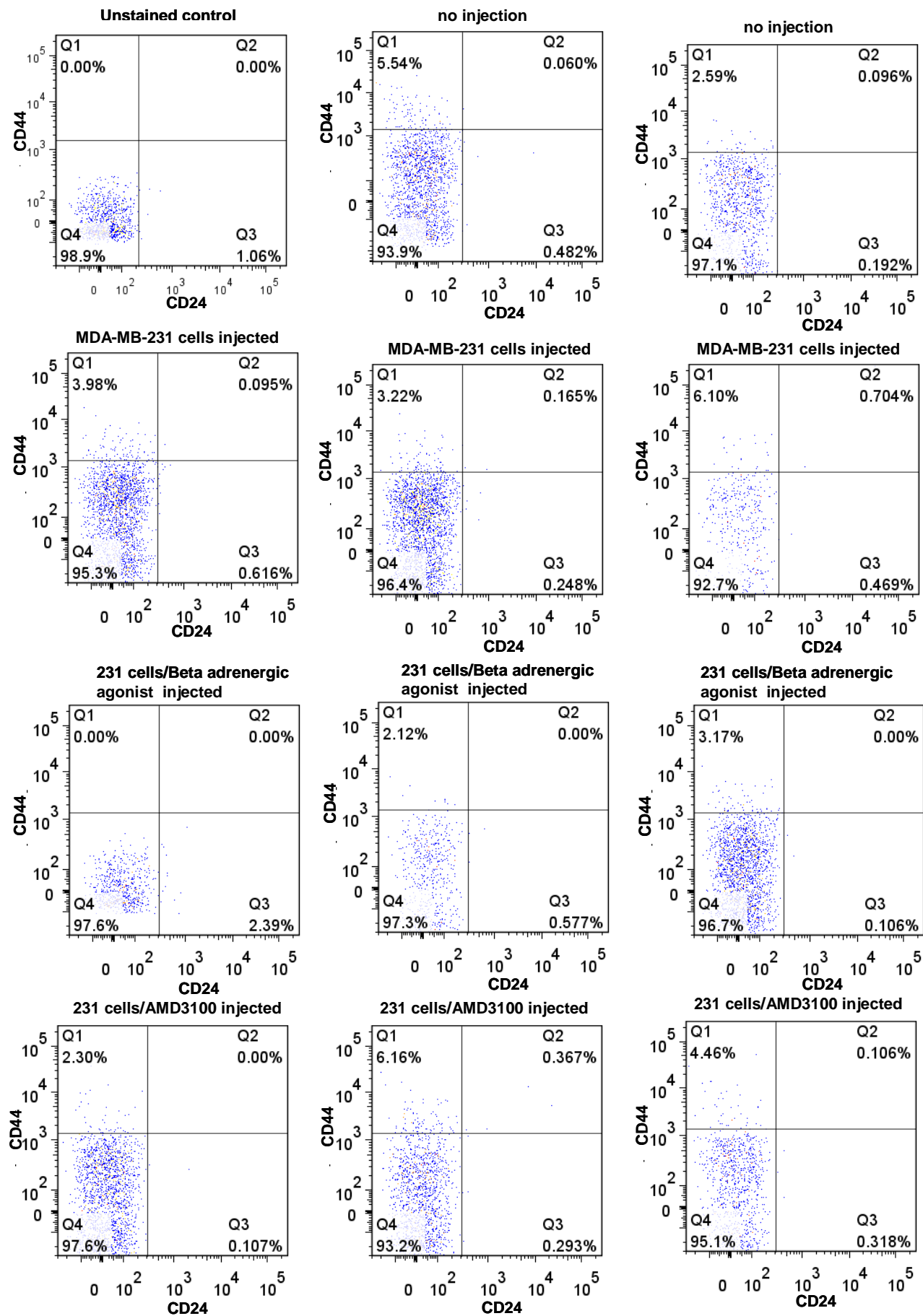
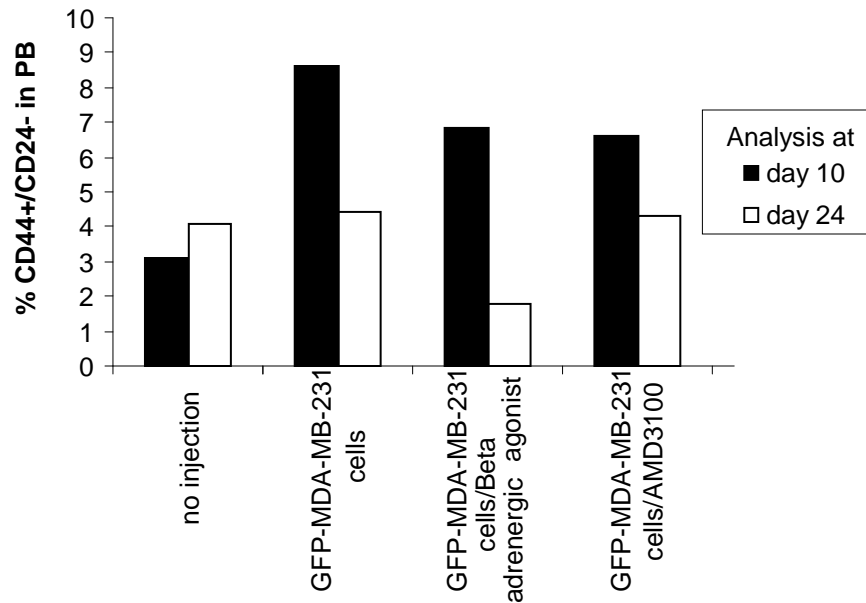


Figure 2

Day 24



**Figure 3**

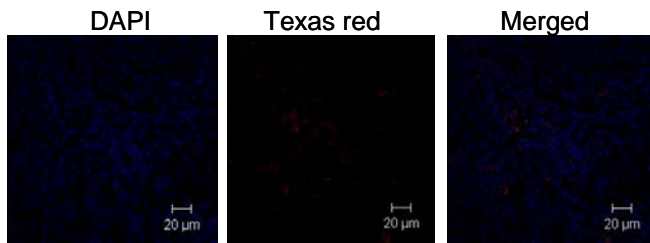
Cells	Day 10 %CD44+/CD24-		Day 24 %CD44+/CD24-	
Treatment	Average	Sd	Average	Sd
No cell injection	3.085	0.75660426	4.065	2.085965
GFP-MDA-MB-231 cells injected into mammary fat pads	8.62	0.91536878	4.433333333	1.49255932
GFP-MDA-MB-231 cells + Beta adrenergic agonist injected	6.813333333	1.12593665	1.763333333	1.61481681
GFP-MDA-MB-231 cells + AMD3100	6.636666667	2.66631456	4.306666667	1.93456283

**Figures 1+2+3:** Female Nude mice were purchased from Jackson Laboratories (Bar Harbor, ME, USA) at 3 weeks of age and were used. Mice received mammary fat pad injection of either MDA-MB-231/GFP cells ( $1 \times 10^6$ ), or no cell injection as control as indicated. Six hours following the time course (10 days and 24 days), mice were received intraperitoneal single injection of either AMD3100 (5mg/Kg) or  $\beta 2$  adrenergic agonist (5mg/Kg). Control mice received i.p. injections of vehicle alone. After six hours, mice were sacrificed, dissected and peripheral blood (PB) was collected. White blood cells (WBC) were isolated by Ficoll separation. Briefly, blood was diluted to 6 ml and was overlaid on the 5 ml Ficoll layer. Tubes were centrifuged at 2000 rpm at 18°C for 30 min. The WBC layer was transferred into a 15 ml conical tube. Wash the cells with PBS once and cells were analyzed for FACS staining. FACS analysis of murine peripheral blood was performed using CD44-PE-CY7 and CD24-PE. All antibodies were from BD biosciences. Flow cytometry analysis was performed on a BD LSRII cell analyzer (BD Bioscience), and data was analyzed by FlowJo 7.6.5 software.

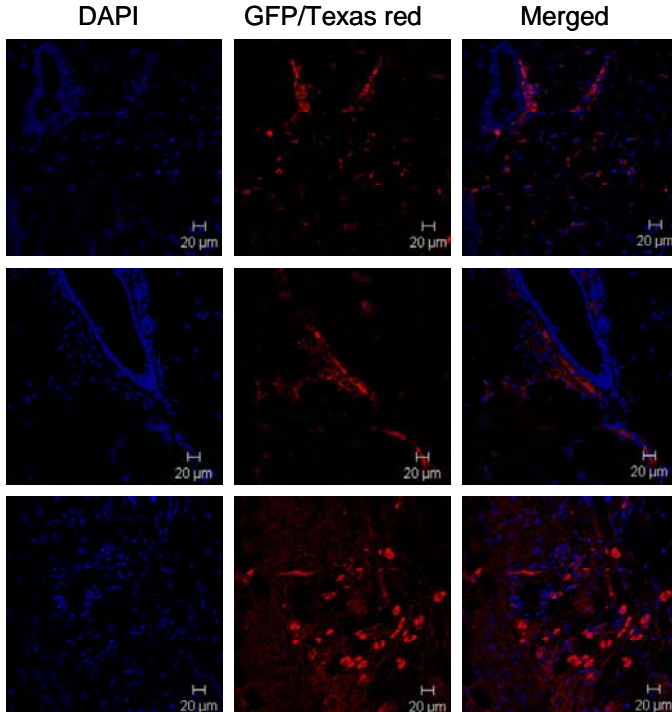


**Figure 4A**

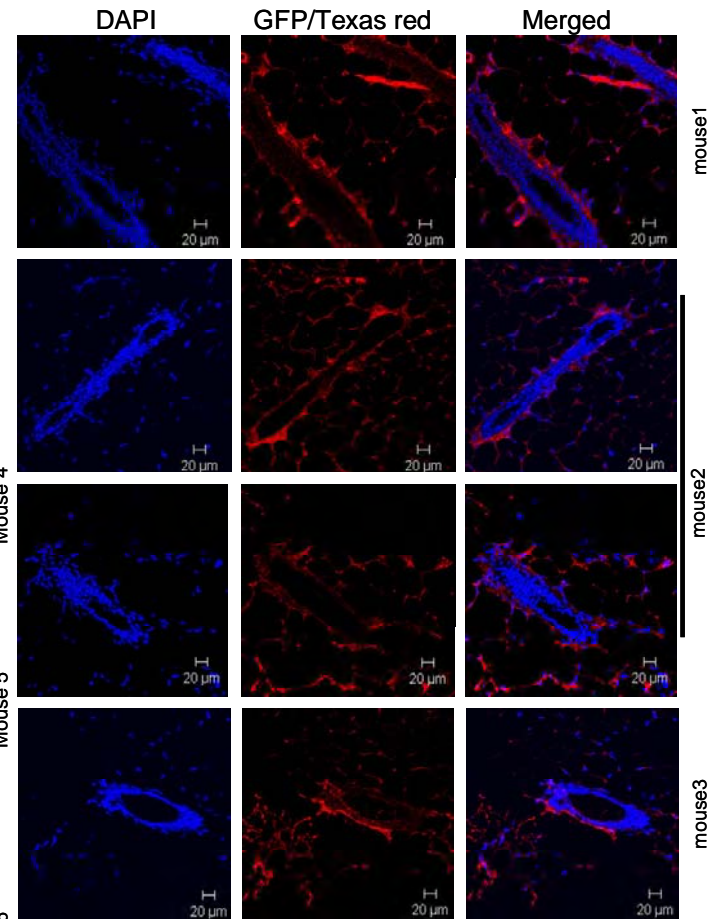
Mammary fat pad – Immunostaining with secondary antibody control

**Figure 4C**

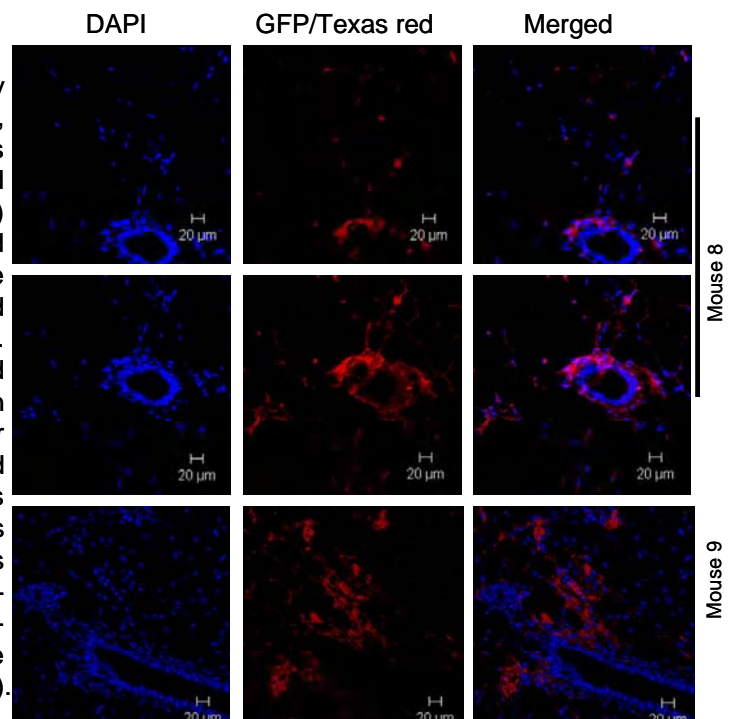
MDA/MB/231GFP cells with beta2 adrenergic agonist

**Figure 4B**

MDA/MB/231GFP cells injected into mammary fat pads

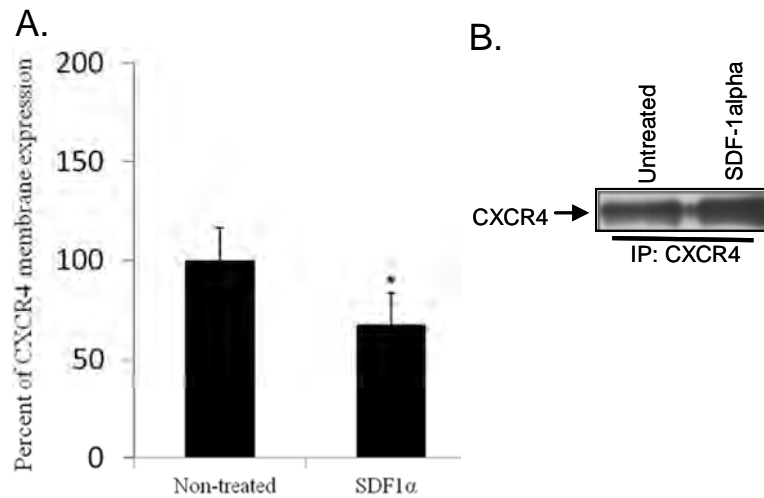
**Figure 4D**

MDA/MB/231GFP cells + AMD3100



**Immunofluorescence Staining:** Mice received mammary fat pad injection of either MDA-MB-231/GFP cells ( $1 \times 10^6$ ), or no cell injection as control as indicated. Six hours following the time course (10 days), mice were received intraperitoneal single injection of either AMD3100 (5mg/Kg) or  $\beta 2$  adrenergic agonist (5mg/Kg). Control mice received i.p. injections of vehicle alone. After six hours mice were sacrificed, dissected and mammary fat pad was collected from each mouse for immunofluorescence stain. Paraffin-embedded sections were deparaffinized and dehydrated, washed in PBS, and subjected to antigen retrieval using treatment with 10mM Citrate Buffer after blocking. Primary antibody staining was performed overnight at 4°C, while secondary antibody staining was performed for 60 minutes and DAPI staining for 5 minutes at room temperature. 1:50 Primary antibody of GFP was used for immunofluorescence. 1:300 of fluorochrome-conjugated secondary antibodies of anti-mouse Ig-Alexa594 (Molecular Probes) was used. Sections were imaged using a confocal microscope (Zeiss LAM 510Meta).

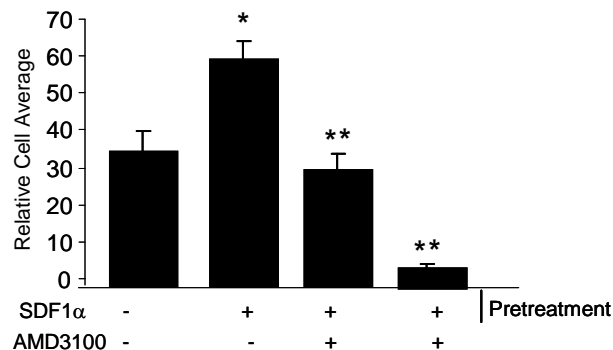
Figure 5



**Figure 5: Activation of CXCR4 inhibits CXCR4 internalization on MDA-MB-231 cells.**

(A) Serum starved MDA-MB-231 cells were treated with 100ng/ml SDF1 $\alpha$  for 10 minutes. Cells were detached and fixed in 4% cold paraformaldehyde for 30 minutes. Cells were washed and blocked prior to incubation with CXCR4-specific antibodies (1:100) at 4° C overnight. CXCR4 was detected with FITC-conjugated secondary antibodies (1:500) by FACS analysis. (B) Serum-starved MDA-MB-231 cells were treated with vehicle or with 100ng/ml SDF1 $\alpha$  for 10 minutes. Lysates (1 mg) were immunoprecipitated with 2 $\mu$ g of anti-CXCR4 antibody overnight at 4°C. Immuno-complexes were analyzed by Western blotting with anti-CXCR4 antibody and detected by enhanced chemiluminescence. \*p<0.005 as compared to control.

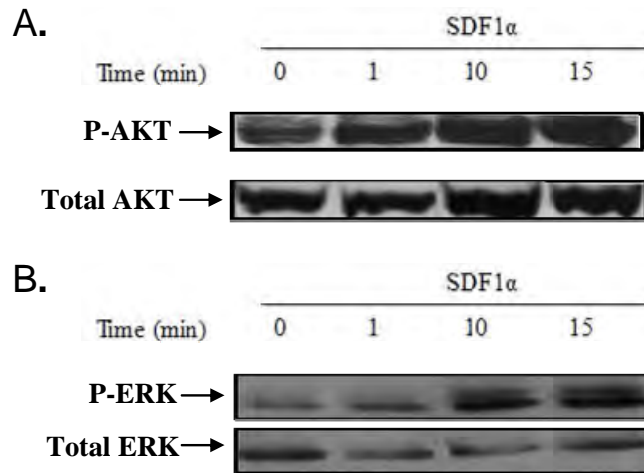
Figure 6



**Figure 6: Activation of CXCR4 increased migratory behavior of MDA-MB-231 cells.**

Serum-starved cells were preincubated with vehicle or combination of SDF1 $\alpha$  (100 ng) and AMD3100 (50 and 100 ng/mL as indicated) for 2 hours, prior to detaching with 1X Citric Saline. 2x10<sup>4</sup> cells each were seeded into the upper well of 24-well transmigration chambers (8.0 $\mu$ m pore; Transwell; Costar), while ligand SDF-1 $\alpha$  was added to the lower chambers. Plates were incubated at 37°C for 4 hours and the inner-upper chambers were cleaned with a cotton swab, while the outer-upper chamber was stained with HEMA 3 System. Stained migrated cells were counted on a light microscope. The migration index was calculated as the x-fold change in migration observed over nontreated cells. \*p<0.05 as compared to control cells. \*\*p<0.005 as compared to SDF-1 $\alpha$  treated cells alone. \*p<0.001 as compared to control.

Figure 7



**Figure 7: Activation by SDF-1 $\alpha$  results in phospho-ERK1/2 and phospho-AKT.**

(A) Serum-starved cells were treated at various time points with SDF1 $\alpha$ . Cells were lysed in 1X CS Lysis Buffer (Cell Signaling), sonicated and separated by SDS-PAGE. Transferred proteins were detected with anti phospho-ERK1/2, anti phospho-AKT, total ERK1/2 or total AKT primary antibodies overnight at 4°C. Bound antibodies were detected by horseradish peroxidase-conjugated secondary antibodies and detected by enhanced chemiluminescence.

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